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RESEARCH ARTICLE

Milk energy output in Swiss mice throughout the first, second, third and fourth lactation events

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SUMMARY

Most studies on the factors limiting sustained energy intake (SusEI) during peak lactation period have been performed in females at the 1st lactation event. However, an inconsistent change in SusEI is observed between the 1st and 2nd lactation event. Thus, the limits to SusEI may be associated with reproductive experiences, but the effects of reproductive experiences on SusEI or reproductive output remain unclear. Here, food intake, reproductive output, suckling behaviour and serum prolactin levels were measured in female Swiss mice throughout the 1st, 2nd, 3rd and 4th lactation periods. Asymptotic food intake was significantly elevated during the 2nd lactation period relative to that observed during the 1st lactation period. Females in the 2nd lactation period exported significantly more energy in milk than those in the 1st lactation event and consequently raised larger litters with heavier litters at weaning. This was inconsistent with the prediction of the peripheral limitation hypothesis, but also did not provide support for the heat dissipation limitation hypothesis. Neither food intake nor reproductive output, indicative of litter size, litter mass and milk energy output (MEO), was different between the 1st, 3rd and 4th lactation event. Differences in suckling behaviour and serum prolactin levels were not significant between the four lactation events. Correlations of prolactin levels with asymptotic food intake, MEO and mammary gland mass were only observed in females during the 1st lactation period. This may suggest that prolactin is not a key factor in stimulating milk production when the mammary glands work at their maximum during the peak lactation period.

Key words: mammary glands, milk energy output (MEO), prolactin, reproductive experience, suckling behaviour, sustained energy intake (SusEI), Swiss mice.

INTRODUCTION

The limitations on sustained energy intake (SusEI) are important because they establish the upper energetic limits on the ability of animals to distribute, survive and reproduce (Peterson et al., 1990; Hammond and Diamond, 1997; Speakman, 2000; Johnson et al., 2001a; Speakman and Król, 2005; Speakman and Król, 2011). The limits to SusEI have previously been suggested to be imposed intrinsically by some aspects of the physiological processes associated with energy intake and/or energy expenditure (Speakman and Król, 2005). The central limitation hypothesis suggests that limitations on SusEI may be imposed by the capacity of the gastrointestinal tract to acquire, process and absorb energy (Weiner, 1992; Hammond and Diamond, 1992; Hammond and Diamond, 1997; Speakman and Król, 2005). In many studies, cold-exposed animals increase their food intake during the peak lactation period beyond the level observed in mice kept at thermoneutral conditions. Total gut length is therefore unlikely to limit SusEI (Hammond et al., 1994; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Król and Speakman, 2003a; Król and Speakman, 2003b). Consequently, additional hypotheses on the potential factors causing limitation, such as the peripheral limitation hypothesis, the heat dissipation limitation (HDL) hypothesis and the saturated neural control hypothesis, have been proposed (Speakman and Król, 2005).

The peripheral limitation hypothesis suggests that SusEI is constrained peripherally by the expenditure capacities of the energy-consuming organs, like the capacity of mammary glands to produce milk during lactation (Hammond and Diamond, 1992; Hammond and Diamond, 1997; Rogowitz, 1998; Hammond and Kristan, 2000; Speakman and Król, 2005; Speakman, 2007; Speakman, 2008). However, inconsistent with the prediction of the peripheral hypothesis, after being exposed to different ambient temperatures (8, 21 or 30°C), MF1 mice show differences in milk production, with more milk being produced at lower temperatures (Johnson and Speakman, 2001; Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2003; Król et al., 2007). This suggests that the capacity to expend energy during the lactation period at 30°C is likely to be constrained by the ability to dissipate heat, whereas cold exposure can be considered a relaxation of the heat dissipation limit, allowing the females to elevate not only their food intake but also their milk production above the heat dissipation limit; that is, the HDL hypothesis (Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005; Speakman and Król, 2010; Speakman and Król, 2011). Dorsal fur removal increases thermal conduction, as well as asymptotic food intake and milk energy output (MEO) in MF1 mice (Król et al., 2007) but has no effect on reproductive output for Swiss mice (Zhao and Cao, 2009; Zhao et al., 2010a). Similarly, Rogowitz (Rogowitz, 1998) found that milk production did not differ significantly between cotton rats (*Sigmodon hispidus*) lactating at 8 and 21°C, which is inconsistent with the HDL hypothesis.

In the context of neuroendocrine mechanisms underlying feeding behaviour, Speakman and Król proposed another hypothesis concerning the limits to SusEI during the lactation period, i.e. the

saturated neural control hypothesis (Speakman and Król, 2005). This hypothesis suggests that food intake is stimulated by a number of hormonal factors in the periphery and the brain, and when receptors become saturated the system cannot be stimulated further. Once the endocrine system is maximally stimulated, females are not capable of increasing asymptotic food intake during peak lactation when they are given more pups or forced to run. Prolactin, known as an important endocrine factor, stimulates milk synthesis in the mammary glands. There is considerable evidence that prolactin is involved in the regulation of food intake. Thus, it may represent a potential new factor associated with neuroendocrine mechanisms underlying limits to SusEI during the lactation period (Noel and Woodside, 1993; Sauve and Woodside, 2000; Bonomo et al., 2005; Speakman and Król, 2005).

Lactation is the most demanding period for female mammals, during which SusEI has been suggested to be constrained by physiology, but the factors limiting SusEI are still not fully understood (Thompson, 1992; Hammond and Diamond, 1997; Rogowitz, 1998; Król et al., 2003; Speakman and Król, 2005; Speakman and Król, 2011). It has previously been reported that changes in litter size or litter mass are observed between the 1st and the 2nd lactation (Oswald and McClure, 1990; Johnson et al., 2001b). In the present study, I examined the difference in reproductive output of Swiss mice throughout the 1st, 2nd, 3rd and 4th lactation periods. Body mass, food intake, litter size and mass, as well as MEO and mammary gland mass were measured. In addition, changes in suckling behaviour and serum prolactin levels were compared between the four lactation events. I aimed to determine different responses, in terms of limits to SusEI, to reproductive experience. I also focused on a potential role of serum prolactin in neuroendocrine mechanisms underlying limits to SusEI during the lactation period. According to the peripheral limitation hypothesis or the HDL hypothesis, I expected that no differences in food intake and reproductive output would be observed throughout the four lactation periods.

MATERIALS AND METHODS

Animals and experimental protocol

Virgin female Swiss mice, 8–10 weeks old, were obtained from a laboratory colony (Experimental Animal Centre of Shandong University, Shandong, China), and were maintained at a temperature of 23±1°C and a photoperiod cycle of 12 h L:12 h D (lights on at 08:00 h). All mice had free access to standard rodent chow (Beijing KeAo Feed Co., Beijing, China) and water. The total energy content of the diet was 17.6 kJ g^{-1}. Prior to the experiment the females were housed individually for at least 1 week in plastic cages (29×18×16 cm) with fresh sawdust bedding.

Female mice (N=104) were paired with males for 11 days and then males were removed. Females were randomly divided into four groups: (1) the 1st lactation event (referred to as L1 hereafter, N=30): 28 females were pregnant and lactating during the 1st lactation period; (2) the 2nd lactation event (L2, N=30): 29 females were pregnant and lactating during both the 1st and 2nd lactation period; (3) the 3rd lactation event (L3, N=22): 17 females succeeded in their pregnancy and parturition during the 1st, 2nd and also the 3rd lactation period, i.e. were allowed to raise young 3 times; and (4) the 4th lactation event (L4, N=22): 20 females were allowed to raise young 4 times. There were 2 week intervals between the day when the litters were weaned in the previous lactation period and the next mating. After parturition (day 0 of the lactation period), litter size and mass were measured on a daily basis, except for days 1–2 of the lactation period. The litters were weaned on day 17 in all groups. Females in L1, L2, L3 and L4 were killed by decapitation on day 17 of the 1st, 2nd, 3rd and 4th lactation period, respectively, for blood and mammary gland sampling.

Body mass and food intake

Female body mass was measured on a daily basis, as was food intake between days 3 and 17 of the lactation period. Food intake was calculated as the mass of food missing from the hopper every day, subtracting orts mixed in the bedding (Johnson et al., 2001a; Johnson et al., 2001c; Zhao and Cao, 2009). As no significant difference in food intake was found between days 9 and 16 of the lactation period by repeated measurements, the asymptotic food intake during the peak lactation period was calculated as the mean daily food intake over this period.

MEO

MEO during the peak lactation period was estimated individually from the energy budget of the litter (E_L) as described previously (Król and Speakman, 2003b), and all estimates of MEO refer to day 14 of the lactation period. The energy budget of the litter was the sum of the energy allocated into respiration (R) and the energy accumulated into new tissues. R was predicted from pup body mass using the relationship between resting metabolic rate (RMR) and body mass. It assumed that R=1.4×RMR to take the energetic costs of pup activity into account. MEO (kJ day^{-1}) was calculated according to Eqn 1 (Król and Speakman, 2003b):

$$\mathrm{MEO} = [(7.28 + 0.71 \times M_L) \times \mathrm{CF_{act}} + M_{L,\mathrm{inc}} \times \mathrm{GE_{pups}}] \times 100 / d_{\mathrm{milk}}, \quad (1)$$

where M_L is litter mass (in g) on day 14 of the lactation period; CF_{act} is the correction factor (1.4); $M_{L,inc}$ is the litter mass increase between days 13 and 14 (in g day^{-1}); GE_{pups} is gross energy content of the pups (in kJ g^{-1}), which was measured from 8 pups using a Parr 1281 oxygen bomb calorimeter (Zhao et al., 2010a); and d_{milk} is the apparent digestibility of milk (96%) (Król and Speakman, 2003b; Oftedal and Iverson, 1987; Zhao et al., 2010a). There were a number of similarities between the MF1 mice and Swiss mice strains, such as the level of RMR during the lactation period, and the mean pup mass and litter mass gain between days 13 and 14 of the lactation period (Johnson et al., 2001c; Zhao et al., 2010a). The equation would therefore be suitable for the estimation of MEO in Swiss mice (Zhao et al., 2010a).

Behavioural observations

Suckling behaviour was observed in 12 female mice with their litters (litter size range 10–13) on days 13–15 of the lactation period. As described previously (Speakman et al., 2001; Zhao et al., 2010a), each cage was observed for 5 s, and a series of 12 cages were observed in sequence for 1 min each. Within the period of behavioural observation, the dominant behaviour was recorded as either suckling or non-suckling for each female. Suckling behaviour was defined as nursing pups in any location either in or outside the nest. If the female did not nurse the pups, no matter what they did or if the pups stopped suckling, the behaviour was defined as non-suckling behaviour. All observations were made during the light phase (08:00 h–20:00 h). Thus, in total, each female was observed 720 times over a period of 12 h. The suckling duration of each mother was calculated as the cumulative suckling behaviour (min $12\,h^{-1}$) (Zhao et al., 2010a).

Serum prolactin

After the 1st, 2nd, 3rd and 4th lactation event, females (from L1, L2, L3 and L4 groups, respectively) were killed immediately by decapitation between 17:00 h and 19:00 h on day 17 of the lactation

period. Trunk blood was collected and serum was separated from each blood sample by centrifugation and stored at –75°C for prolactin measurement. Serum prolactin levels were quantified by radioimmunoassay using RIA kits (Beijing North Biological Technical Research Institute, Beijing, China). This RIA kit was validated and used for Swiss mice following the standard kit instructions (Zhao et al., 2010a). Intra- and inter-assay coefficients of variation were less than 10% for prolactin.

Mammary glands

After blood was collected, females were immediately dissected. All mammary glands were carefully removed from each female and pooled. The mammary glands were weighed to 0.001 g to determine wet mass, dried in an oven at 60±1°C for 10 days to a constant mass, and then weighed (to 0.001 g) again to determine dry mass.

Statistical analysis

Statistical analysis was carried out with the SPSS13.0 software package. Repeated measures ANOVA was used to examine the changes in female body mass and food intake, as well as litter size and mass over the 1st, 2nd, 3rd and 4th lactation periods. Differences in body mass and food intake of females, litter size and mass, as well as MEO, suckling behaviour, serum prolactin and mammary gland mass between L1, L2, L3 and L4 groups were examined using one-way ANOVA, followed by Tukey's *post hoc* test. Pearson correlation analyses were used to detect possible correlations of asymptotic food intake with litter size, litter mass, MEO and mammary gland mass. All data are expressed as means ± s.e.m. Statistical significance was taken at $P<0.05$.

RESULTS

Body mass

Body mass was significantly different between groups during the early lactation period (day 3: L1, 49.4±0.4 g; L2, 55.1±0.6 g; L3, 57.1±0.6 g; L4, 63.3±0.9 g; $F_{3,213}=86.2$, $P<0.001$; Fig. 1A). L4 females had significantly higher body mass than L1, L2 and L3 females (*post hoc*, $P<0.05$). Body mass of L2 and L3 females was significantly higher than that of L1 females. The difference between L2 and L3 females was not significant (*post hoc*, $P>0.05$). Differences in mass consistent with the difference in body mass on day 3 were observed throughout the lactation period (day 17, $F_{3,213}=86.2$, $P<0.001$; Fig. 1A).

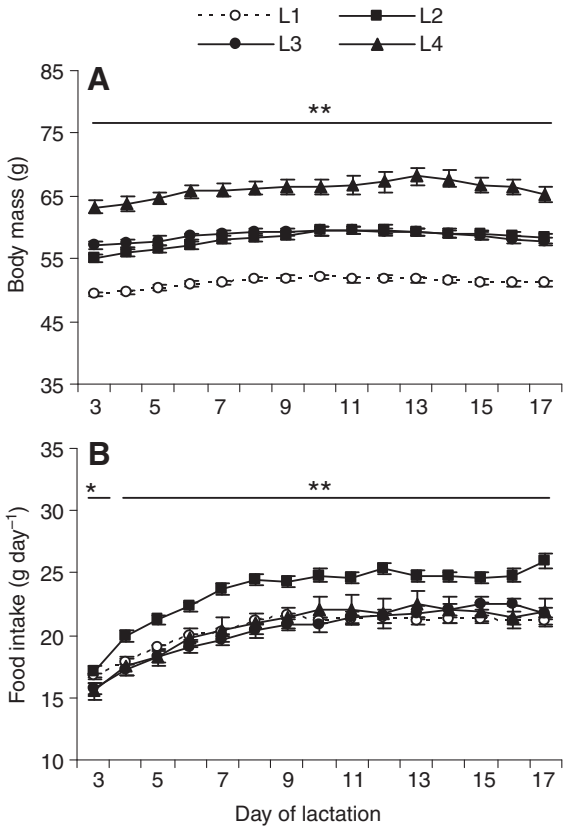

Fig. 1. Body mass (A) and food intake (B) of females throughout the 1st, 2nd, 3rd and 4th lactation period (L1, L2, L3 and L4) in Swiss mice. Data are means ± s.e.m. Asterisks indicate a significant difference between the four groups (*$P<0.05$, **$P<0.01$).

Food intake

There was a significant difference in food intake between the four groups on any day throughout lactation (day 3, $F_{3,213}=3.1$, $P<0.05$; day 17, $F_{3,213}=21.2$, $P<0.01$; Fig. 1B). Food intake of L2 females was significantly higher than that observed in the other three groups both early (day 4, *post hoc*, $P<0.05$) and late in the lactation period (day 17, *post hoc*, $P<0.05$). No difference in food consumption was found between L1, L2 and L3 females (day 4, *post hoc*, $P>0.05$; day 17, *post hoc*, $P>0.05$). The asymptotic food intake averaged 374.6±3.9, 435.7±6.7, 381.6±8.4 and 385.7±16.5 kJ day^{-1} for L1, L2, L3 and L4 females and was significantly different between the groups ($F_{3,213}=21.0$, $P<0.001$). L2 females had 16.3, 14.2 and 13.0% higher asymptotic food intake than L1, L3 and L4 females, respectively (*post hoc*, $P<0.05$), whereas food intake was similar in L1, L3, L4 females (*post hoc*, $P>0.05$).

Litter size

Litter sizes were significantly different between the groups (day 3, $F_{3,213}=9.6$, $P<0.01$; Fig. 2A) and averaged 10.9±0.2, 12.8±0.3, 10.8±0.5 and 10.2±0.8 in L1, L2, L3 and L4 females. Females in L2 raised significantly larger (heavier) litters than those in L1, L3 or L4 (day 3, *post hoc*, $P<0.05$). Litter size was not different between L1, L3 and L4 groups (day 3, *post hoc*, $P>0.05$). There was a significant difference in litter size between the four groups on any day throughout lactation, and the litters were weaned with 10.0, 12.4, 10.6 and 9.9 pups for L1, L2, L3 and L4 females (day 17, $F_{3,213}=13.3$, $P<0.01$; Fig. 2A). Litter size was positively correlated with asymptotic food intake for L1, L2, L3 and L4 females (Fig. 3A).

Litter mass

On day 3 of the lactation period, litter mass averaged 31.9±0.9 g in L2 females, which was 14.9, 19.8 and 22.9% higher than that in L1, L3 and L4 females ($F_{3,213}=8.7$, $P<0.01$; *post hoc*, $P<0.05$; Fig. 2B). Litter mass was not significantly different between L1, L3 and L4 groups on day 3 (*post hoc*, $P>0.05$). On any day of the lactation period, the difference in litter mass was significant between the four groups, and litters were weaned at a mass of 81.5±1.3, 104.1±1.7, 89.0±2.5 and 85.8±4.8 g for L1, L2, L3 and L4 groups, respectively (day 17, $F_{3,213}=33.4$, $P<0.01$). On day 17, litter mass in L2 females was heavier by 27.8, 16.9 and 21.3% than that observed for L1, L3 and L4, respectively (*post hoc*, $P<0.05$). No differences in weaning mass were observed between L1, L3 and L4 females (*post hoc*, $P>0.05$; Fig. 2B). I observed a positive correlation between litter mass and asymptotic food intake for L1, L2, L3 and L4 females (Fig. 3B).

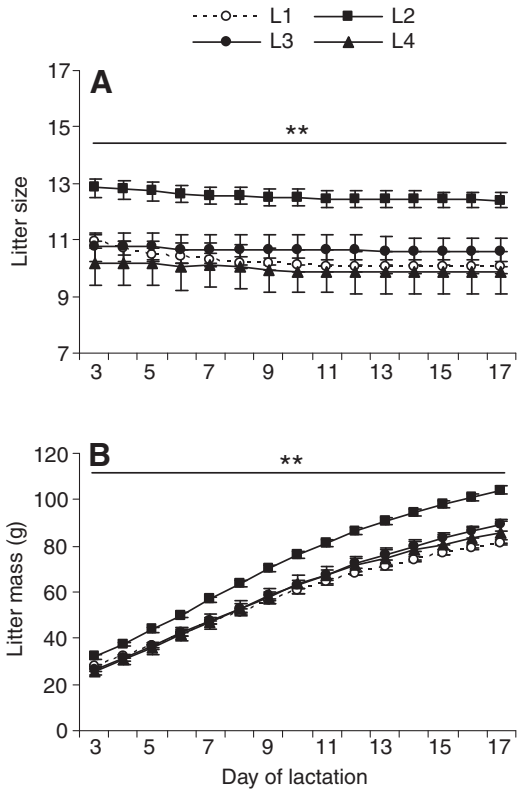

Fig. 2. Litter size (A) and litter mass (B) throughout L1, L2, L3 and L4 in Swiss mice. Data are means ± s.e.m. **Significant difference between the four groups (P<0.01).

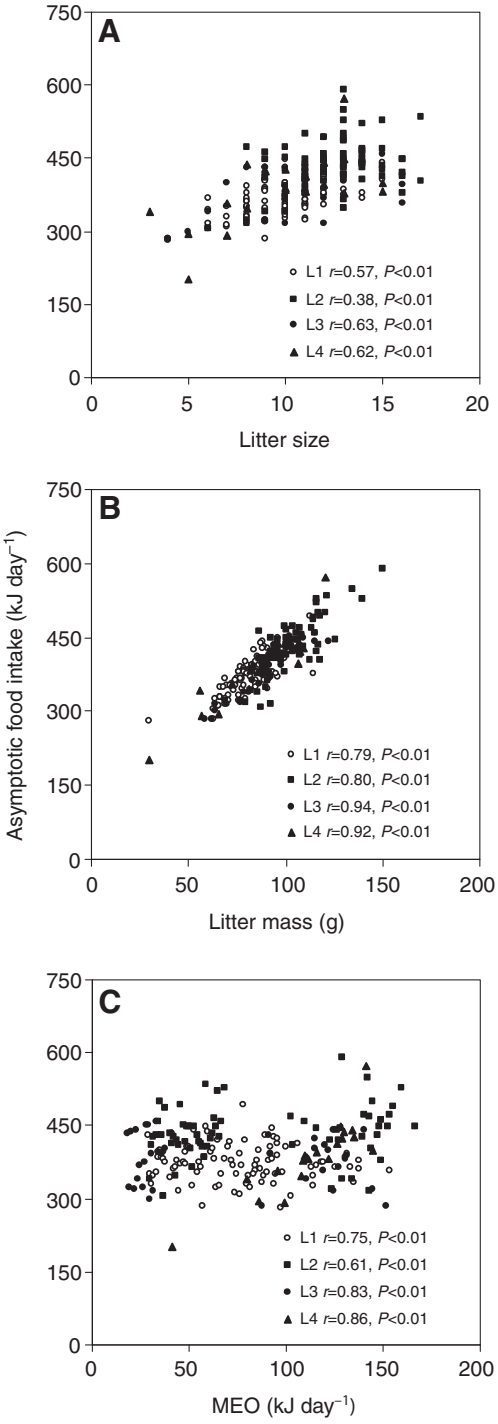

Fig. 3. Correlations between asymptotic food intake and litter size (A), litter mass (B) and milk energy output (MEO, C) throughout L1, L2, L3 and L4 in Swiss mice.

MEO

MEO was significantly different between the four groups ($F_{3,90}$=10.0, P<0.01, Fig. 4A). MEO was 25.4, 13.5 and 21.1% higher in L2 females than in L1, L3 and L4 females (*post hoc*, P<0.05). No differences in MEO were observed between L1, L3 and L4 females (*post hoc*, P>0.05). MEO was positively correlated with asymptotic food intake for L1, L2, L3 and L4 groups (Fig. 3C). There were also significantly positive correlations between MEO and litter size, as well as litter mass for the four groups (Fig. 5A,B).

Mammary glands

I observed significant differences in the mass of the mammary glands between the four lactation periods ($F_{3,90}$=10.0, P<0.01, Fig. 4B). The highest mammary gland mass was found in L2 females; it was 72.5% higher than that in L1 females (*post hoc*, P<0.05) and 21.6% higher than that in L3 females (*post hoc*, P<0.05). Yet, L2 and L4 females did not differ in mammary gland mass (*post hoc*, P>0.05). Mammary gland mass was not different between L3 and L4 groups (*post hoc*, P>0.05), but both were significantly heavier than that of L1 group (*post hoc*, P<0.05). Positive correlations between mammary gland mass and MEO were observed in L1, L2, L3 and L4 groups (Fig. 5C).

Suckling behaviour and serum prolactin levels

L1 females showed similar suckling bouts to L2, L3 and L4 females (Table 1). Neither cumulative suckling duration within 12 h nor mean suckling duration differed between the four groups. There was also no difference in serum prolactin levels between L1, L2, L3 and L4 groups (Table 1). Serum prolactin levels were significantly positively correlated with asymptotic food intake, MEO and mammary gland mass for L1 females, but this correlation was not observed in L2, L3 and L4 females (Fig. 6).

DISCUSSION

Lactation is the most demanding period that female mammals have to cope with. During this high energy demanding period, the female

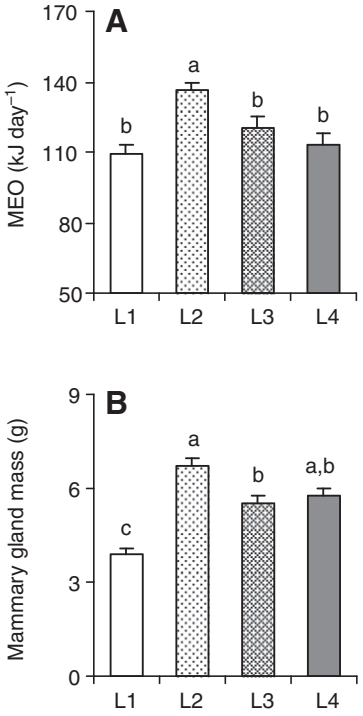

Fig. 4. MEO (A) and mammary gland mass (B) throughout L1, L2, L3 and L4 in Swiss mice. Data are means ± s.e.m. Different letters above columns (a, b or c) indicate significant differences between the four groups (P<0.05).

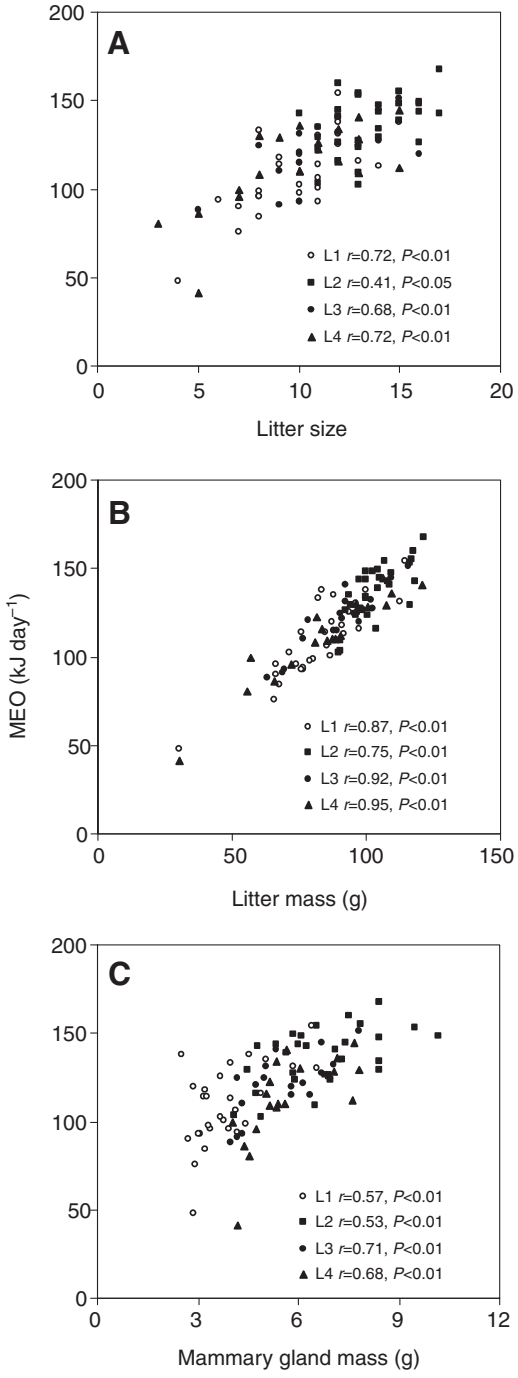

Fig. 5. Correlations between MEO and litter size (A), litter mass (B) and mammary gland mass (C) throughout L1, L2, L3 and L4 in Swiss mice.

invests not only in the offspring but also in her own soma (Speakman, 2008). In the present study, I observed significant differences in female body mass throughout the lactation period for L1–L4. Body mass was higher in L2 and L3 than in L1, but was lower than that in L4. Additionally, L2 females had significantly higher food intake and reproductive output than the other three groups. The differences of body mass and some possible consequent effects for reproduction are of interest, but the reasons remain uncertain. There are three possible explanations for the differences. The first is that females gain in mass after they complete the 2nd and 3rd lactation events because there is a 2 week interval before they are mated for the next reproduction. Second, L2 females have heavier mammary glands, which also contribute to the body mass gain. The third explanation is that L2 females have higher food consumption compared with L1, L3 and L4 females. Thus L2 females require only minor adjustments in morphology of the alimentary tracts and associated organs including the liver and the pancreas (Kennedy et al., 1958; Jolicoeur et al., 1980; Hammond, 1997; Speakman, 2008), resulting in apparently higher body mass. However, the alimentary tract and associated organs require greater maintenance costs (Krebs, 1950), which would impact on food intake and lactation performance (Speakman, 2008).

An animal usually increases energy intake to meet the most energetically demanding periods such as lactation (Karasov, 1986; Hammond and Diamond, 1997; Johnson et al., 2001a; Thompson and Nicol, 2002; Speakman and Król, 2005; Speakman, 2007; Zhang and Wang, 2007). In the present study, Swiss mice increased their food intake throughout the 1st lactation period, but reached a ceiling around 21 g day^{-1} during the late lactation period, which was consistent with previous studies on the same strain of mice (Hammond and Diamond, 1992; Hammond and Diamond, 1994; Hammond et al., 1994; Hammond and Diamond, 1997; Zhao and Cao, 2009; Zhao et al., 2010a). The limits to SusEI have previously been suggested to be imposed by the capacity of an animal to dissipate heat or by the ability of the mammary glands to produce milk, or possibly both (Hammond and Diamond, 1992; Hammond et al., 1996; Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005; Król et al., 2007; Zhao and Cao, 2009; Speakman and Król, 2010; Zhao et al., 2010a). Here, I compared asymptotic food intake during the peak lactation period in females raising young

Table 1. Suckling behaviour and serum prolactin levels in Swiss mice throughout the 1st, 2nd, 3rd and 4th lactation events (L1, L2, L3 and L4, respectively)

	L1	L2	L3	L4	*F*	*P*
Litter size range	8–13	10–16	8–13	8–13		
Mean litter size	10.3±0.4[b]	13.1±0.5[a]	10.3±0.4[b]	10.6±0.5[b]	8.4	**
Suckling bouts (12 h)	9.3±0.5	10.1±0.8	10.3±0.5	9.4±0.7	0.7	n.s.
Cumulative suckling duration (min 12 h^{-1})	455.2±26.6	473.6±28.8	469.0±23.8	445.5±27.8	0.2	n.s.
Mean suckling duration (min)	49.6±1.6	48.8±2.8	46.1±1.3	48.2±2.2	0.5	n.s.
Serum prolactin levels (U ml^{-1})	128.8±7.6	135.2±11.7	121.0±8.1	116.7±10.5	0.7	n.s.

Values are presented as means ± s.e.m. n.s., non-significant difference between groups (P>0.05). **P<0.01. Different letters on the same row (a or b) indicate significant differences (P<0.05).

throughout four consecutive events. I found that females in L2 had ~16, ~14 and ~13% higher asymptotic food intake than females in L1, L3 and L4. A significant increase in asymptotic food intake was also observed in MF1 mice in L2 compared with those in LI (Johnson et al., 2001b). This indicated that asymptotic food intake during the 2nd lactation period exceeded the limit set during the 1st lactation event. As the thermal environment did not change over the period of four lactations, the females were assumed to face similar heat dissipation conditions. This finding was inconsistent with the prediction of the HDL hypothesis.

An increase in energy intake during peak lactation is likely to support the high energy demand of the gastrointestinal system as well as other organs involved in milk production such as the mammary glands (Rogowitz, 1998; Zhao et al., 2010b). Consistently, I found significant correlations between asymptotic food intake and reproductive output indicative of litter size, litter mass and MEO in Swiss mice. I also observed that the females in the 2nd lactation period exported significantly higher energy by milk secretion than the mothers in the 1st lactation event. According to the HDL hypothesis, an animal was constrained by the capacity to dissipate heat and thus failed to further increase food intake and reproductive output if heat dissipation was unchanged (Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005; Król et al., 2007; Speakman and Król, 2010; Speakman and Król, 2011). I predicted that no change in heat dissipation should occur between the different lactation events. However, I found that MEO increased further in L2 females than in L1 females so heat dissipation limitation could not be confirmed.

Another hypothesis associated with limits to SusEI, called the peripheral limitation hypothesis, suggests that the mammary glands operate maximally during late lactation and consequently impose limits on SusEI (Hammond and Diamond, 1992; Hammond and Diamond, 1994; Hammond et al., 1996; Hammond and Diamond, 1997; Rogowitz, 1998; Hammond and Kristan, 2000; Speakman and Król, 2005; Zhao et al., 2010a). The pups depend entirely on milk. In the present study, I found positive correlations of MEO with litter size and litter mass, as well as a significant relationship between MEO and mammary gland mass. I also observed a further increase in MEO in the females in the 2nd lactation period in parallel with asymptotic food intake, as well as litter size and mass. This might suggest that the function of the mammary glands developed physiologically during the 2nd lactation period, producing more milk, which was then considered to go beyond the maximum levels set during the 1st lactation period. Conceivably, these data were not consistent with the peripheral limitation hypothesis. Johnson and colleagues also reported similar data in MF1 mice, within which the females gave birth to significantly more pups and raised significantly heavier litters at weaning in the 2nd lactation period than in the 1st (Johnson et al., 2001b).

In addition to the work in laboratory mice, the studies regarding to the limits to SusEI have been performed in wild-captured animals, some of which strongly support the peripheral limitation hypothesis based on the synthetic capacity of the mammary glands. For example, levels of milk production are similar between cotton rats lactating at 21 and 8°C (Rogowitz, 1998). Milk production does not change in rabbits (*Oryctolagus cunniculus*) and captive mink (*Mustela vision*) raising different litters (Drummond et al., 2000; Fink et al., 2001). The data from other species provide support for the HDL theory, such as the dramatic increase in energy intake observed in the cold compared with the warm in cotton rats (Rogowitz, 1998), deer mice [*Peromyscus maniculatus* (Hammond and Kristan, 2000)], Brandt's voles [*Lasiopodomys brandtii* (Zhang and Wang, 2007)] and striped hamsters [*Cricetulus barabensis* (Zhao, 2011)]. It has been further shown that if lactating Brandt's voles are exposed to hot conditions (30°C), then their intake and milk energy output are limited at a much lower level (Wu et al., 2009). Domesticated rabbits under hot conditions have impaired reproductive performance (Marai et al., 2001). These results may suggest that there is a species-specific response to the limits to SusEI. Either peripheral limits or heat dissipation limits are observed in some species, but may not be found in others.

The inconsistent data have also been observed in the same species between different strains or even in a same animal under different conditions. For instance, the data from Swiss mice are consistent with the prediction of the peripheral limitation hypothesis (Hammond and Diamond, 1994; Hammond et al., 1996; Zhao and Cao, 2009; Zhao et al., 2010a), but the studies in MF1 mice provide strong support for the HDL theory (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2007). Exposure of European hares (*Lepus europaeus*) to temperature conditions of 5°C during lactation leads to a massive increase in energy intake and milk transfer to young, supporting the HDL hypothesis (Valencak et al., 2010). However, the hares at thermoneutrality rearing cold-exposed young are able to increase energy intake to levels indistinguishable from those of cold-exposed females, inconsistent with the HDL hypothesis (Valencak et al., 2010). The inconsistency may indicate that an animal is constrained both by the capacity of mammary glands to secret milk and the ability to dissipate body heat, but the two limits are set at different levels (Speakman and Król, 2011).

In the previous studies in Swiss mice, limits on SusEI are probably more consistent with the peripheral limitation hypothesis, but do not strongly refute the HDL theory (Hammond and Diamond, 1994; Hammond et al., 1996; Zhao and Cao, 2009; Zhao et al., 2010a). From the present study, the mice showed a difference in energy budget between the four lactations, suggesting that the limits may also be affected by the reproductive experiences. The seasonal investment hypothesis suggests that seasonally reproducing rodents generally perceive a high reproductive value of offspring born early

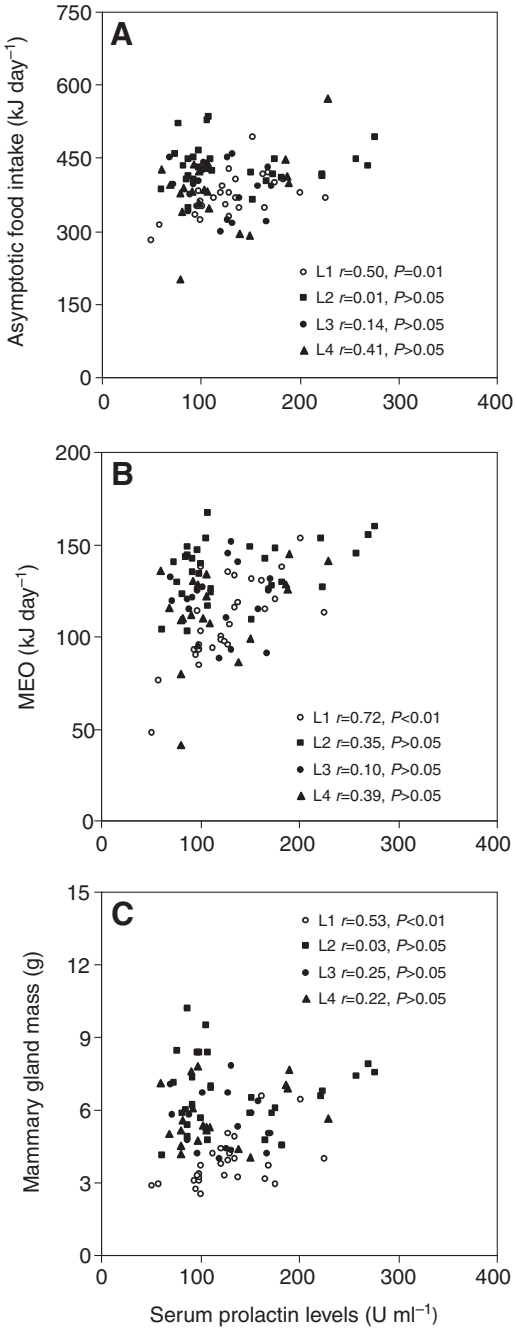

Fig. 6. Correlations between serum prolactin levels and asymptotic food intake (A), MEO (B) and mammary gland mass (C) throughout L1, L2, L3 and L4 in Swiss mice.

in the season and a much lower reproductive value of offspring born late (Speakman and Król, 2005; Lambin and Yoccoz, 2001). It has also been observed that there is a trade-off between maternal and offspring energy requirements (Rogowitz, 1998). Animals do not reproduce maximally because this reduces the probability of their own survival (Rogowitz, 1998; Speakman and Król, 2005). Here, I observed that both energy intake and reproductive output are higher in the 2nd lactation but lower in the following 3rd and the 4th lactation. This may suggest that laboratory mice can also perceive a difference in the reproductive value of offspring between lactations, and consequently invest more in offspring during the 2nd lactation but invest less in the 3rd and the 4th lactation. It may also indicate that the trade-off between maternal and offspring energy requirements is different between lactations.

Prolactin plays an important role in stimulating milk synthesis in the mammary gland (Speakman and Król, 2005). Here, serum prolactin levels were positively correlated with mammary gland mass, as well as with asymptotic food intake and MEO during the 1st lactation period. Thus, serum prolactin levels are likely to be involved in the regulation of food intake during the peak lactation period and become a potential factor involved in limits to SusEI, a concept which is consistent with the prediction of the saturated neural control hypothesis (Sauve and Woodside, 2000; Bonomo et al., 2005; Speakman and Król, 2005; Zhao et al., 2010a). However, in the present study such significant correlations were not observed in the females during the 2nd, 3rd or 4th lactation periods. In addition, suckling bouts and cumulative suckling duration were not different between the four lactation events, although the females raised significantly larger litters in the 2nd lactation period. Female mice have 10 teats and, assuming that whenever the litter is suckling all the teats are occupied, the females may receive the same stimulus from a litter of 10 as from a litter of more than 10 (Johnson et al., 2001a). This was probably the reason why there was similar suckling behaviour between the four lactation periods (Johnson et al., 2001a). The females had hyperplastic mammary glands and an increased MEO in the 2nd compared with the first lactation event, but failed to upregulate serum prolactin levels. This might suggest that prolactin was not a key factor stimulating milk production when the mammary glands worked at their maximum during the peak lactation period. It might also indicate that an animal did not increase circulatory prolactin because all prolactin receptors were saturated (Farmer et al., 1999). However, from the current study the neuroendocrine mechanisms by which MEO increased further in the 2nd lactation period were not fully understood. Injection of prolactin during lactation might be helpful for further testing the role of prolactin in the regulation of milk production and other functions. Other hormonal signals in the periphery and the brain would also be involved, like leptin, insulin, neuropeptide Y (NPY), Agouti-related peptide (AgRP), etc. (Speakman and Król, 2005), but unfortunately were not measured in my study.

CONCLUSIONS

In the present study, significant changes in asymptotic food intake and reproductive output were observed in female mice between four lactation events. Asymptotic food intake was significantly elevated in the 2nd lactation event relative to the asymptotic level observed during the 1st lactation period. Females exported significantly more energy into milk during the 2nd lactation period and consequently raised larger and heavier litters until weaning. This was inconsistent with the prediction of the peripheral limitation hypothesis, but also did not provide support for the HDL hypothesis. I failed to find any difference between the 1st, 3rd and 4th lactation events. Neither suckling behaviour nor serum prolactin levels were different for L1–L4. Correlations of prolactin with asymptotic food intake, MEO and mammary gland mass were observed in L1 females only, which may suggest that prolactin is not a key factor stimulating milk production when the mammary glands work at their maximum during the peak lactation period. Thus, measurement of other hormonal signals in the periphery and the brain would also be needed to examine the saturated neural control hypothesis.

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